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RNase III Participates in GadY-Dependent Cleavage of the *gadX-gadW* mRNA

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The adjacent *gadX* and *gadW* genes encode transcription regulators that are part of a complex regulatory circuit controlling the *Escherichia coli* response to acid stress. We previously showed that the small RNA GadY positively regulates *gadX* mRNA levels. The *gadY* gene is located directly downstream of the *gadX* coding sequence on the opposite strand of the chromosome. We now report that *gadX* is transcribed in an operon with *gadW*, although this full-length mRNA does not accumulate. Base pairing of the GadY small RNA with the intergenic region of the *gadX-gadW* mRNA results in directed processing events within the region of complementarity. The resulting two halves of the cleaved mRNA accumulate to much higher levels than the unprocessed mRNA. We examined the ribonucleases required for this processing, and found that multiple enzymes are involved in the GadY-directed cleavage including the double-strand RNA-specific endoribonuclease RNase III.

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Introduction

Regulatory RNAs in organisms from all kingdoms act by a wide array of mechanisms that enable them to play major roles in controlling gene expression. In bacteria, these regulatory RNAs are typically small in size (50–300 nt) and therefore have been termed small RNAs (sRNAs). Much of what is currently known about sRNA function in bacteria has arisen from studies of the Gram-negative model organism *Escherichia coli* in which around 80 sRNAs have been identified.[1] While not all of these sRNAs have been characterized, great strides have been made in understanding their mechanisms of action (reviewed in Ref. 2). The largest class of sRNAs elicits regulatory effects through the formation of base-pairing interactions with target mRNAs. Many of these sRNAs are encoded in *trans* with respect to their target mRNAs and contain only limited complementarity with the targets. In these cases, the RNA binding protein Hfq is usually required for sRNA function. Approximately one third of all *Escherichia coli* sRNAs bind to Hfq, and it is thought that each of these sRNAs acts by base pairing with *trans*-encoded mRNA targets. Four major regulatory outcomes have been established for the Hfq-dependent RNAs: inhibition or activation of translation or increased or decreased degradation of target mRNAs.

A limited number of base-pairing sRNAs that are encoded on the strand opposite annotated genes on bacterial chromosomes, and thus in *cis* to their

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Abbreviations used: sRNA, small RNA; UTR, untranslated region; RACE, rapid amplification of cDNA ends.

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targets, have also been characterized.[3] Due to this genetic arrangement, the sRNA and the target mRNA have the potential for extensive base pairing across the region of overlap. In these cases, the Hfq protein generally has not been found to be required for base pairing. Most of the *cis*-encoded sRNAs with known functions negatively regulate translation and promote the degradation of the complementary mRNA.

We have been characterizing the 105-nt *E. coli* GadY RNA, which is encoded in *cis* to the 3′ end of the *gadX* gene in the ~371-nt intergenic region between *gadX* and *gadW* (Fig. 1a). Intriguingly, this sRNA was found to positively regulate *gadX* mRNA levels.[4] The *gadX* mRNA encodes a transcription regulator that is involved in a highly complex regulatory circuit controlling the response to acid stress.[5,6] The *gadW* gene located immediately downstream from *gadX* also encodes a regulator of the acid response and can be transcribed with *gadX* or from its own promoter as an independent transcript.[5–8] We confirm here that *gadX* is transcribed as a two-gene mRNA with *gadW*. When the GadY sRNA is present, the *gadX-gadW* mRNA is processed to give rise to products with ends within the region complementary to GadY. Part of this processing is due to the GadY-dependent RNase III cleavage. However, GadY pairing with the *gadX-gadW* mRNA also modulates cleavage by other ribonucleases in mutants lacking RNase III.

Results

Region of complementarity confers GadY-dependent regulation on a heterologous gene

We previously showed that the GadY RNA positively regulates *gadX*, and that this regulation requires sequences in the 3′ untranslated region (UTR) of the *gadX* mRNA[4] (see also Fig. 1b). The 3′-UTR sequences of *gadX* needed for regulation are perfectly complementary to GadY due to the *cis*-encoded nature of these two RNAs, and it was proposed that base pairing within this region is necessary for regulation to occur.

We further investigated the mechanism of regulation by testing if the GadY RNA was capable of regulating a heterologous gene tagged with the 3′ UTR of *gadX*. A hybrid gene was constructed by replacing the *gadX* coding sequence with the coding sequence of the chloramphenicol resistance gene *cat*, such that the sequences downstream of the *gadX* coding sequence will be expressed as part of the *cat* mRNA (Fig. 1c). In addition, the *gadX* promoter was replaced with the *cat* promoter to eliminate the wild-type transcriptional regulation. To abolish the expression of the chromosomally encoded GadY RNA, the *gadY* promoter mutation described previously (TATATT -10 sequence replaced by GGGGGG) was also introduced into the strain. We then examined the levels of *cat* mRNA without and with GadY overexpression from a plasmid (Fig. 1d). The Northern blots showed that the *gadX* 3′ UTR is sufficient to confer GadY-dependent regulation on the heterologous *cat* gene. The strain carrying the pRI control vector had low levels of a band corresponding to the expected ~0.8 kb size of the *cat* mRNA. Multiple faint bands corresponding to longer transcripts that hybridized with the *cat* gene probe were also observed for this control strain. In contrast, only the ~0.8-kb *cat* mRNA was detected in the strain overexpressing GadY, and this transcript was present at much higher levels.

GadY RNA directs processing

The longer *cat* transcripts observed in the absence of GadY suggested that transcription of the reporter was proceeding through *cat* to downstream sequences. In the presence of the GadY RNA, these possible read-through transcripts were no longer detected. To confirm the read-through transcription, we made an additional modification to the *cat* reporter construct. A promoterless *gfp* gene encoding the green fluorescent protein was inserted into the chromosome downstream from *cat* and separated from the *cat* gene by the sequences complementary to GadY such that the native promoters for *gadW* and *gadY* were eliminated. The 3′ UTR of *gadX*, encompassing the region of base pairing with GadY, remained intact. The *gfp* gene was also engineered to contain *rrn* terminators downstream from the coding sequence so that any transcripts reading through *gfp* would be forced to terminate (Fig. 1e). We assayed for *gfp* expression in the absence or presence of GadY by examining cells under a fluorescent microscope. Unexpectedly, the reporter strain was fluorescent under both conditions, indicating that transcription initiating at the *cat* promoter was proceeding through *gfp* regardless of GadY expression (data not shown). We then examined each gene in the reporter operon by Northern blot analysis in strains carrying the control vector or overexpressing GadY. When GadY was absent, a single hybridizing mRNA was detected when the Northern blots were probed with the *cat* specific probe (Fig. 1f). The size of this mRNA (~1.7 kb) was consistent with a two-gene transcript encompassing both *cat* and *gfp* (Fig. 1e). When GadY was overexpressed, a single hybridizing mRNA was detected, but now the size of the transcript (~0.8 kb) was consistent with a single gene mRNA encompassing only the *cat* gene (Fig. 1e). Similar results were seen when the same total RNA samples were probed with a *gfp*-specific oligonucleotide. In the absence of the

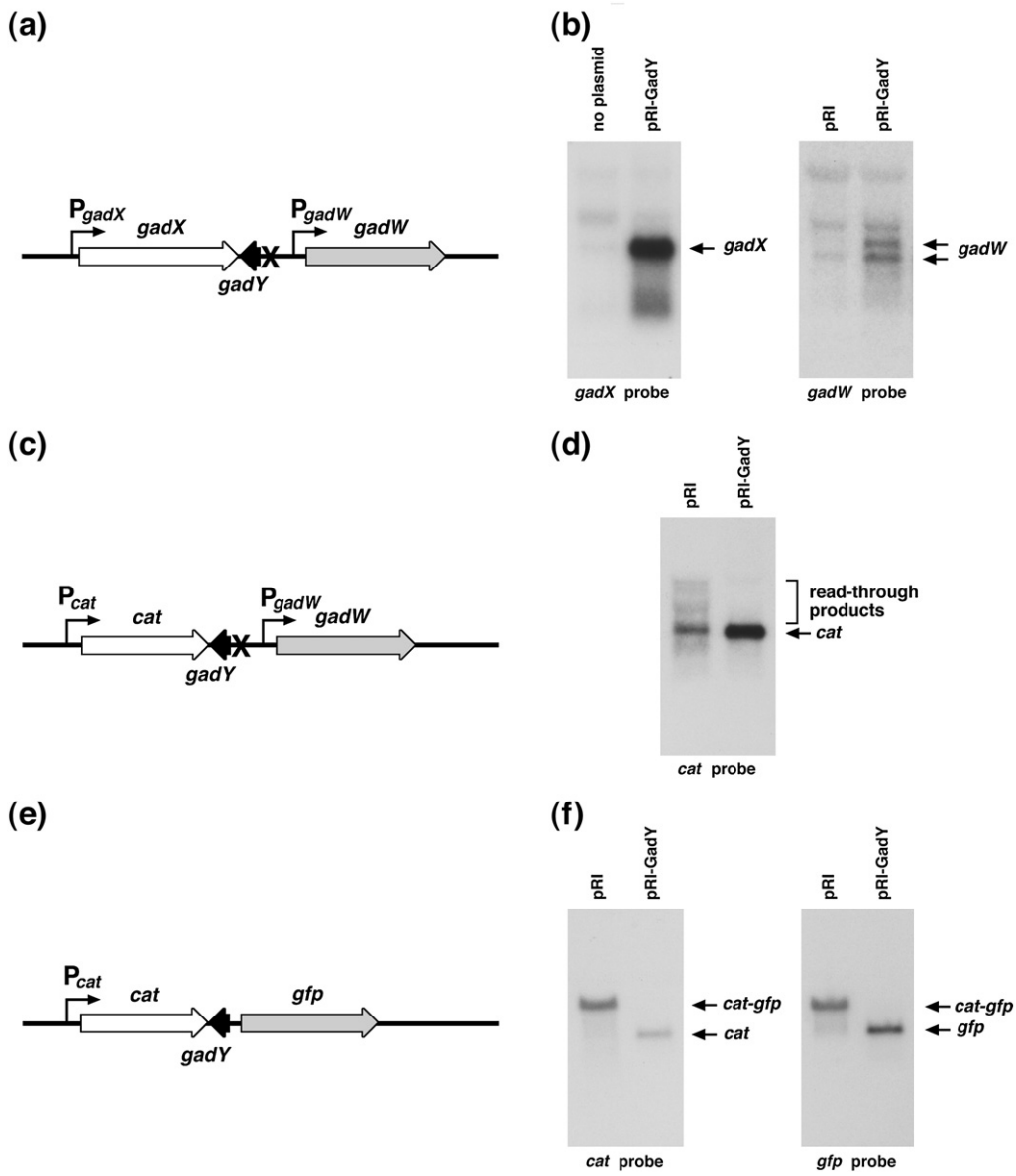

Fig. 1. GadY RNA-directed mRNA cleavage. (a) Diagram of the *gadXWY* region. The *gadY* promoter mutation is indicated by X. (b) Levels of the *gadX* (~1.0 kb) and *gadW* (~1.1 kb) mRNAs in cells without a plasmid, carrying the vector control (pRI), or overexpressing GadY (pRI-GadY). The top band in both panels of (b) is likely to be due to cross-hybridization with the 16S rRNA. (c) Diagram of the *cat* replacement of *gadX*. (d) Levels of the *cat* (~0.8 kb) mRNA in cells carrying the vector control (pRI) or overexpressing GadY (pRI-GadY) in a background (GSO129) in which the *gadX* promoter and coding sequence were replaced with the *cat* promoter and coding sequence. (e) Diagram of the *cat* replacement of *gadX* and *gfp* replacement of *gadW*. (f) Levels of the *cat* (~0.8 kb, ~1.7 kb) and *gfp* (~0.9 kb, ~1.7 kb) mRNAs in cells carrying the vector control (pRI) or overexpressing GadY (pRI-GadY) in a background (GSO403) in which the *gadX* promoter and coding sequence were replaced with the *cat* promoter and coding sequence and a promoterless *gfp* gene was inserted downstream and separated from *cat* by the sequences complementary to GadY. In all cases, total RNA (5 μg each) isolated from cultures grown in LB medium to $OD_{600} = 0.7$ was separated in a 1% agarose–0.05 M Mops–1 mM EDTA gel and then transferred onto a nylon membrane. The individual mRNAs were detected by specific oligonucleotides (gadX-R, gadW-A2, cat-A1, and gfp-R).

GadY RNA, a longer mRNA encompassing both *cat* and *gfp* was detected, while expression of GadY resulted in a smaller transcript (~0.9 kb) encompassing only *gfp* (Fig. 1f). These results suggest that expression of GadY causes a processing event in the mRNA intergenic region between the two reporter genes.

To test whether the GadY sRNA also was directing the processing of the wild-type *gadX-gadW* mRNA, we performed Northern blot analysis

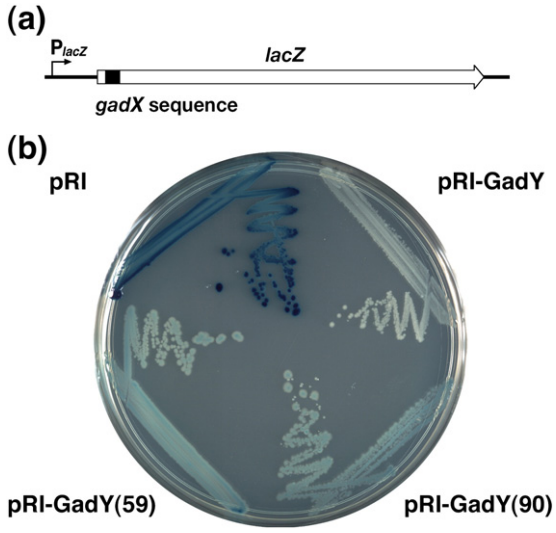

Fig. 2. (a) Map of the $lacZ_{gadX}$ reporter. (b) Plate with $lacZ_{gadX}$ reporter strain (GSO404) harboring pRI, pRI-GadY, pRI-GadY(90), or pRI-GadY(59). The strains were streaked on an LB plate containing 100 μg/ml X-gal. At $OD_{600}=0.7$, the levels of β-galactosidase activity in the strains harboring pRI and pRI-GadY were 320 and 0.3 Miller units, respectively (data not shown).

with a probe to detect *gadW* in a strain with the chromosomal *gadY* promoter mutation transformed with the control vector or the GadY overexpression plasmid (Fig. 1b). In the absence of GadY overexpression, we saw low levels of a *gadW* mRNA (lower band) whose size (~1.1 kb) corresponds to a transcript originating from the previously identified *gadW*-specific P1 promoter,[7] which we confirmed by primer extension analysis (data not shown). When GadY was overexpressed, higher levels of the first transcript were detected together with a second slightly larger transcript. The size of the slightly larger mRNA (~1.5 kb) is consistent with the *gadW* half of a *gadX-gadW* transcript that could be obtained upon GadY-dependent processing. The increase in the lower band could also be the result of increased *gadW* transcription due to increased GadX levels or could correspond to other *gadX-gadW* fragments derived from processing. We note that we did not detect a clear band corresponding to the full-length *gadX-gadW* mRNA (~2.3 kb) in the presence or absence of the GadY RNA, indicating that the two fragments resulting from processing are capable of accumulating to higher levels than the unprocessed mRNA.

To establish an assay in which the effects of GadY could easily be detected, we also constructed a reporter strain in which the 105-nt *gadX* 3′-UTR sequence complementary to GadY was placed in-frame in the amino-terminal portion of the *lacZ* coding sequence in the *E. coli* chromosome (Fig. 2a). The *gadX* 3′-UTR sequence, which is not normally translated, contained at least one nonsense codon in each of the three possible frames. Therefore, a stop codon was eliminated from one of the frames by introduction of an A→G transition. This single mutation maintains base-pairing potential, since the introduced G residue is capable of pairing with the corresponding U nucleotide in wild-type GadY. Our expectation was that in the absence of GadY, the full-length *lacZ* mRNA would be generated, allowing the production of β-galactosidase, while in the presence of GadY, the mRNA would be cleaved, preventing the production of β-galactosidase. The GadY sRNA was previously shown to exist in three stable forms: a full-length 105-nt form and two processed fragments of 90 and 59 nt.[4] We tested the ability of all three forms of GadY to regulate this reporter gene by examining β-galactosidase activity on indicator plates containing X-gal (Fig. 2b). Colonies of this reporter strain were blue in the absence of GadY, but were white when full-length GadY was overexpressed and slightly blue when GadY(90) or GadY(59) was overexpressed. These results are consistent with the conclusion that all three forms of GadY direct the processing of the *lacZ* reporter mRNA.

Processing sites are within the region of complementarity

GadY-dependent processing was examined more precisely by carrying out primer extension analysis to identify the 5′ ends of the *gadX-gadW* cleavage products in a strain carrying the *gadY* promoter mutation (Fig. 3a). We also mapped the 5′ ends of the

Fig. 3. All three forms of GadY direct the processing of its complementary sequence. (a) Primer extension analysis to map the 5′ ends of the processed *gadX-gadW* mRNA. (b) Primer extension analysis to map the 5′ ends of the processed $lacZ_{gadX}$ reporter mRNA. For both (a) and (b), primer extension assays were performed with total RNA isolated from strains GSO109 and GSO404 carrying pRI, pRI-GadY, pRI-GadY(90), and pRI-GadY(59) and grown to $OD_{600}=0.7$ in LB medium using oligonucleotides GadW-A2 and lacZ-R2, respectively. The same oligonucleotides were used to prime the adjacent sequences from plac-$gadX_{gadY\text{-}10}$ mutant and pACYC-$lacZ_{gadX}$. Black bars indicate the extent of the different GadY transcripts. (c) Positions of cleavage. The sequences of base-paired *gadX-gadW* mRNA and GadY RNA are given. The 5′ ends of the three forms of the GadY RNA are labeled and indicated by small boxes. Arrows and larger box denote the Rho-independent terminator. Red arrows indicate the GadY-dependent 5′ ends; blue arrows indicate the GadY(90)-dependent 5′ ends; and green arrows indicate the pRI-GadY(59)-dependent 5′ ends. Larger arrows indicate the most intense bands. The band that decreased in *rnc* mutant strains (see Fig. 4) is indicated by an asterisk.

cleavage products in the *lacZ* reporter strain (Fig. 3b). Although a few bands were different between the two strains, the majority of the 5′ ends resulting from GadY-dependent processing were identical between *gadX-gadW* and the *lacZ* reporter. The strains constitutively overexpressing the full-length GadY

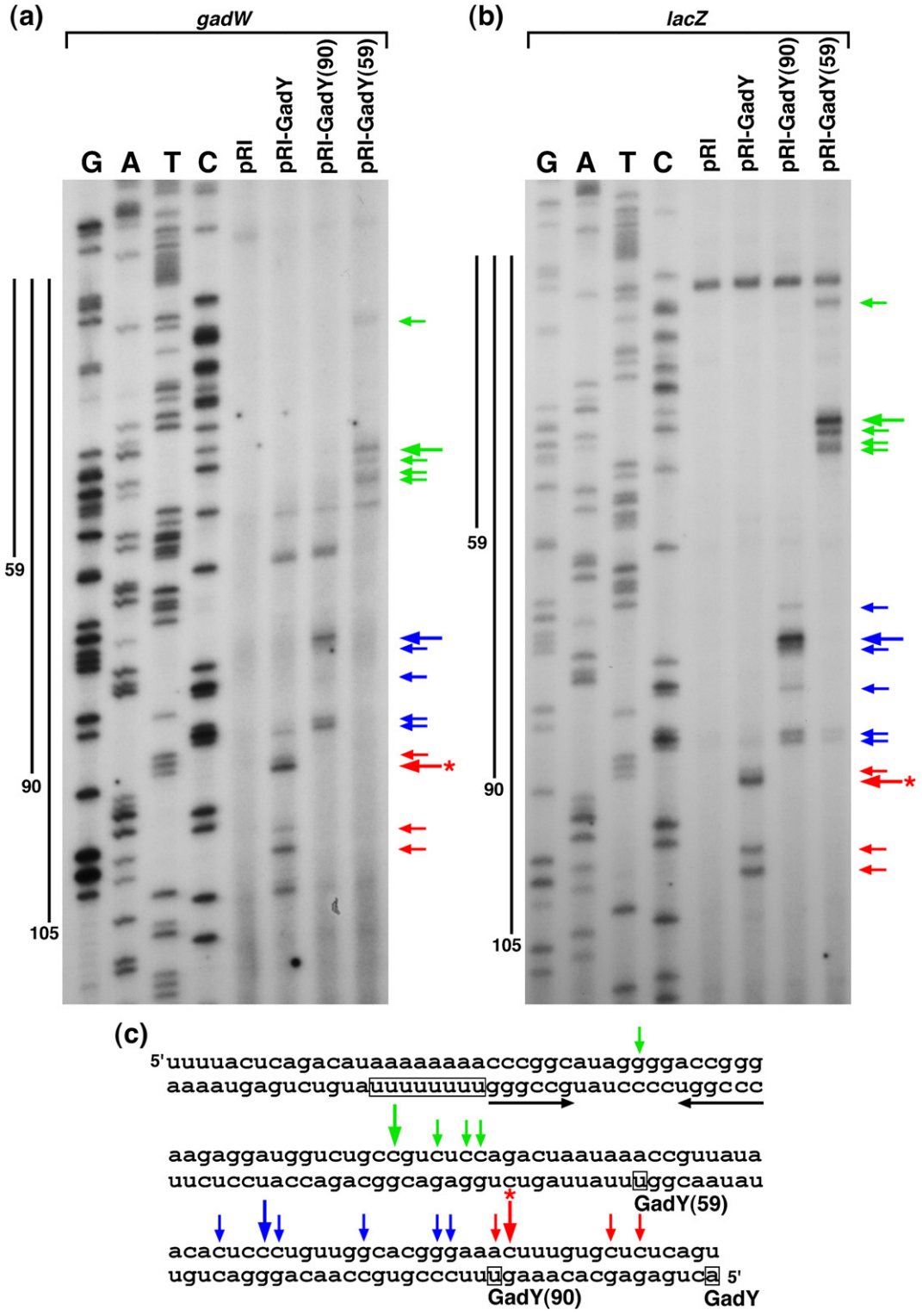

Fig. 3 (*legend on previous page*)

RNA displayed four of the same products. The relative intensity of the bands varied somewhat between experiments, but all corresponded to 5′ ends within the region of base pairing with GadY. These 5′ ends are within 1–3 nt of the *gadW* ends, denoted P3 and P4 by Tramonti *et al.*[7] We also tested the ability of the two shorter forms of GadY to affect processing. Both the 90- and 59-nt forms of GadY led to processing of the *gadX-gadW* mRNA and the *lacZ* reporter at multiple nucleotides, but interestingly, the pattern of primer extension products observed is different for each form of GadY. In each case, most of the 5′ ends of the products correspond to cleavage sites within the region of base pairing near the 5′ end of that particular form of GadY.

Processing is not abolished in endoribonuclease mutants

Given that the same cleavage products were observed for the $lacZ_{gadX}$ construct and the native *gadX-gadW* and that the *lacZ* products were easier to detect, we utilized the reporter strain to test the roles of five endoribonucleases in *E. coli* (reviewed in Ref. 9) in GadY-dependent processing. We first assayed strains carrying single mutations in the genes encoding RNase III (*rnc*), RNase E (*rne*), RNase G (*rng*), RNase BN (*elaC*), and RNase P (*rnpA*). Strains lacking the nonessential ribonucleases, *rnc* (RNase III), *rng* (RNase G), and *elaC* (RNase BN), were grown under conditions that allowed constitutive expression of the *lacZ* reporter. Expression of the three different forms of GadY was induced for 20 min from the arabinose-inducible P_{BAD} promoter, whereupon the 5′ ends of the *lacZ* reporter mRNA were mapped by primer extension (Fig. 4 and data not shown). GadY-dependent processing in the *rng* and *elaC* mutants was identical to the wild strain for all three versions of GadY, although an additional band was observed for all samples, including the vector control strain, for the *rng* mutant. Although processing was not eliminated in the *rnc* mutant, the level of one band was decreased for strains overexpressing full-length GadY (indicated by an asterisk). We used a modified protocol to test the effects of temperature-sensitive mutations in the essential *rne* and *rnpA* genes encoding RNase E and the protein component of the RNase P complex, respectively. In this assay, the temperature-sensitive mutant strains were grown at the permissive temperature under conditions that promoted constitutive expression of the reporter gene. The strains were then shifted to the nonpermissive temperature for 30 min to deplete the cells of active RNase E or RNase P. GadY was then induced for 20 min, while the cells remained at the nonpermissive temperature, and total RNA isolated from these samples was examined by primer extension. Neither of these mutations had an effect on GadY direct processing

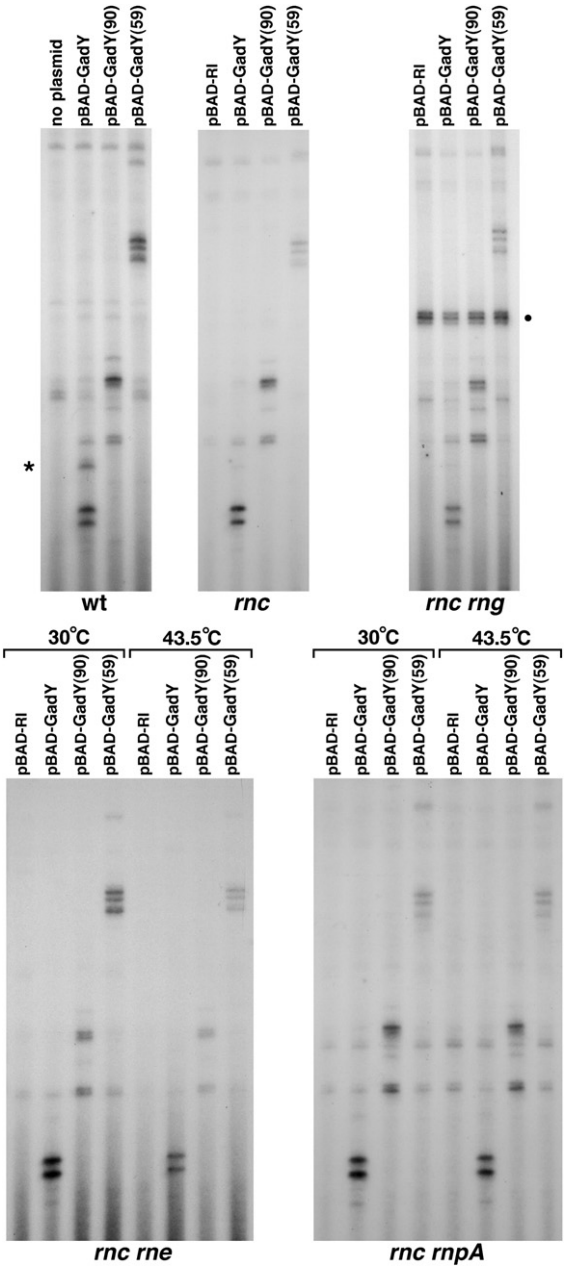

Fig. 4. GadY-directed processing in RNase mutant strains. Total RNA was isolated from wild-type and RNase mutant (*rnc*, *rnc rng*, *rnc rne*, *rnc rnpA*) derivatives of the $lacZ_{gadX}$ reporter strain harboring pBAD-RI, pBAD-GadY, pBAD-GadY(90), or pBAD-GadY(59), 20 min after the addition of arabinose to a final concentration of 0.2% to actively growing cultures (OD 600 ≈ 0.7). For the strains carrying the temperature-sensitive *rne* and *rnpA* alleles, half of the culture was shifted to the nonpermissive temperature (43.5 °C) for 30 min prior to induction with arabinose. Primer extension analysis was performed using labeled oligonucleotide lacZ-R2, and the products were separated on a 6% polyacrylamide gel. The band that decreased in *rnc* mutant strains is indicated by an asterisk, and the extra band present in *rng* mutant strains is indicated by a bullet.

of our reporter gene (data not shown). In addition, we examined the effects of GadY-dependent processing in strains carrying different combinations of these mutations, including several double mutants and the triple-mutant strains *rnc rne rng*, *rnc rne elaC*, *rnc rng elaC*, and *rne rng elaC*. The *rnc* mutation was also combined with a mutation in the *rnlA* gene encoding the RNase LS endonuclease. We did not detect any effects on GadY-dependent processing beyond the band decreased in *rnc* mutants (Fig. 4, Fig. S1, and data not shown).

In vitro processing of *gadX*

Since processing was not abolished in the endonuclease mutant strains, we established an *in vitro* assay to determine whether GadY itself was catalytic or whether a protein was required for the GadY-dependent cleavage. For this assay, a 300-nt *gadX* mRNA fragment containing the entire sequence complementary to GadY was synthesized *in vitro* and labeled throughout its length with ^{32}P (Fig. 5a). This *gadX* mRNA fragment, preincubated with or without unlabeled *in vitro* synthesized GadY RNA, was mixed with a protein lysate. When GadY was added in the absence of lysate, no cleavage was detected (Fig. 5b, lane 2), suggesting that GadY itself was not catalytic and that an additional factor was needed. When only the lysate was added to the reaction, the *gadX* transcript was almost completely digested (Fig 5b, lane 3). However, when both GadY and the lysate were added, a specific processed fragment of *gadX* was detected (Fig 5b, lane 4). The size of this GadY-dependent fragment (~140 nt) suggested that it could correspond to the 5′ portion of the *gadX* mRNA fragment. Although only the 140-nt fragment was visible in Fig. 5b, additional GadY-dependent fragments centered around 100 nt in size corresponding to the 3′ portion of the *gadX* mRNA were detected when the reaction products were analyzed by primer extension analysis (data not shown). The fragments were not generated when the protein lysate was heat treated prior to incubation in the reaction, indicating that the factor required for GadY-dependent processing is likely a protein (Fig. 5b, lane 6).

RNase III is partially responsible for GadY-dependent processing *in vitro*

We then set out to purify the processing activity from an *E. coli* lysate. A six-step purification process was established with the activity monitored at each purification step (see Materials and Methods). After the third purification step, we detected a second major RNA fragment of 100 nt in the *in vitro* processing reaction, in addition to the 140-nt fragment detected when the assay was carried out with the lysate. The size of the smaller fragment corresponds to the expected size of the 3′ *gadX* fragment detected by the primer extension assay

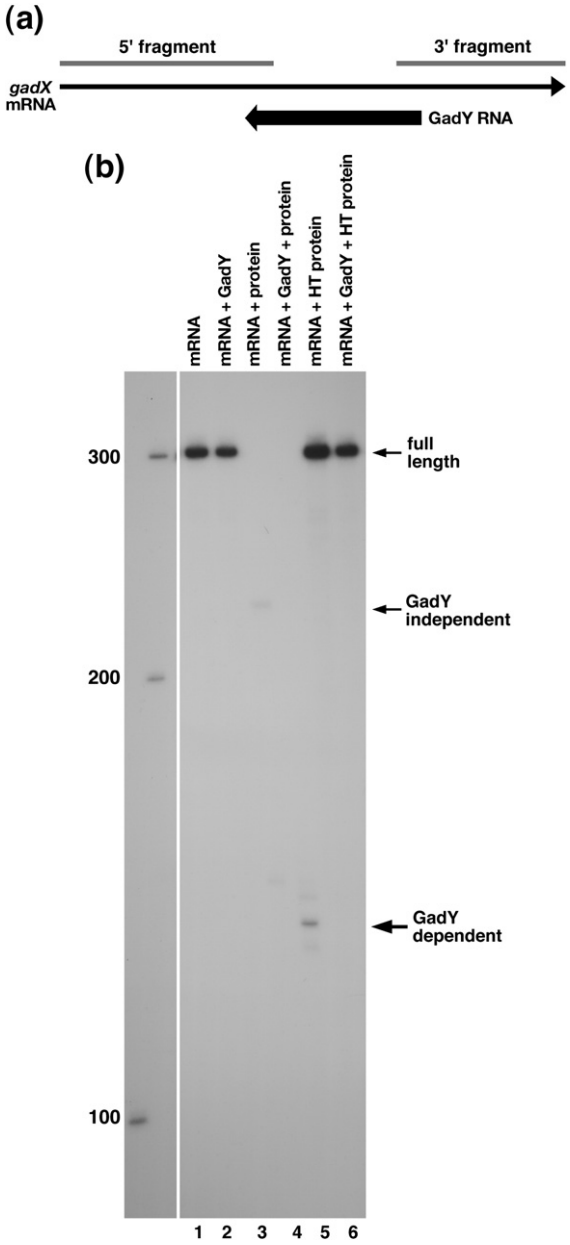

Fig. 5. GadY can direct the processing of *gadX in vitro*. (a) Diagram of the *gadX* and GadY constructs used for the *in vitro* assay. Black arrows indicate the *in vitro* synthesized *gadX* and GadY RNAs. Gray bars indicate the 140-nt 5′ fragment and 100-nt 3′ fragment detected when the *gadX* RNA was processing in the presence of RNase III. (b) A ^{32}P-labeled *gadX* mRNA fragment (mRNA) was incubated at 37 °C for 1 h with the indicated combinations of *in vitro* synthesized GadY sRNA (GadY), GSO430 crude cell extract (protein), and heat-treated GSO430 crude cell extract (HT protein). Treated samples were separated on a 6% polyacrylamide gel alongside a Perfect Marker RNA ladder.

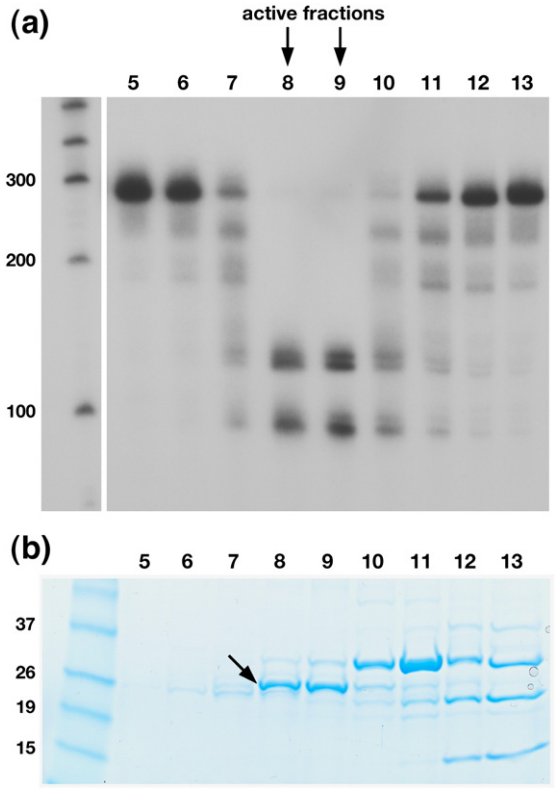

Fig. 6. RNase III is partially responsible for GadY-directed processing. (a) Processing activity from column fractions from final purification step. The ^{32}P-labeled *gadX* mRNA fragment was incubated with GadY and 5 μl of Mono S column fractions for 1 h at 37 °C. Treated samples were separated on a 6% polyacrylamide gel alongside a Perfect Marker RNA ladder. (b) TCA-precipitated protein from column fractions from the final purification step were separated on a 10–20% Tris–glycine SDS-PAGE gel and stained with GelCode Blue. The protein band, indicated by an arrow, was excised from the gel and analyzed by mass spectrometry.

carried out for the lysate sample (see above). We attribute the appearance of this second fragment to increased stability resulting from the elimination of exonucleases during purification. The activity assays and protein profile for the column fractions for the last purification step are shown in Fig. 6. The protein band that co-purified with the activity was excised from the gel and analyzed by mass spectrometry. This analysis revealed that the co-purifying protein was the double-strand specific endoribonuclease RNase III.

Fragments yielded by GadY-dependent RNase III processing

The *in vitro* processing assay with our partially purified RNase III protein yielded two fragments of 140 and 100 nt, which do not add up to the size of the full-length 300-nt *gadX* mRNA template. Primer extension analysis showed that the 100-nt fragment corresponds to the 3′ portion of the processed RNA, with the most prominent primer extension product corresponding to the *rnc*-dependent band observed for *in vivo* cleavage products (Fig. S2). Other bands detected in our primer extension assays carried out on the products generated by the purified fraction correspond to other 5′ ends mapped *in vivo*, as well as three nearby nucleotides. To more accurately define the 140-nt fragment, we also carried out 3′ rapid amplification of cDNA ends (RACE) analysis (Fig. S2). The *gadX* mRNA that was treated with our purified RNase III in the absence of GadY had a 3′ end that mapped to the terminal 3′ end of the *in vitro* synthesized full-length *gadX* RNA. However, when full-length GadY was added to the reaction, two different 3′ ends separated by 4 nt were identified. These ends were centered around 140 nt downstream from the terminal 5′ end of the *in vitro* synthesized *gadX* RNA, in agreement with the 140-nt fragment detected. Other fragments that correspond to the central region of the *gadX* fragment, which may or may not be degraded, were not detected by our primer extension and 3′ RACE analysis.

Figure 3 showed that all three forms of GadY could direct processing, although the 5′ ends generated are different. To test whether our *in vitro* assay mimics the *in vivo* results, we also mapped the 5′ and 3′ ends of the fragments generated by our purified RNase III fraction when GadY90 and GadY59 were added to the reaction. The inclusion of the two shorter forms of GadY in the *in vitro* assay resulted in essentially the same GadY(90)- and GadY(59)-specific 5′ ends detected by primer extension for the *in vivo* products, with some additional nearby ends (Fig. S2). The 3′ ends corresponding to the 140-nt fragment generated by GadY90 and GadY59 were identical to those observed for full-length

Fig. 7. GadY-directed processing in the absence of RNase III. (a) Plate with *lacZ* reporter strain harboring pRI or pRI-GadY in wild-type and *rnc* mutant backgrounds (GSO404 and GSO405, respectively). (b) Levels of the *cat* (~0.8 kb, 1.7 kb) and *gfp* (~0.9 kb, ~1.7 kb) mRNAs in cells carrying the vector control (pRI) or overexpressing GadY in wild-type and *rnc* mutant backgrounds (GSO403 and GSO432, respectively). (c) Primer extension analysis of *gfp* transcripts in cells carrying the vector control (pRI) or overexpressing GadY in wild-type and *rnc* mutant backgrounds (GSO403 and GSO432, respectively). (d) A ^{32}P-labeled *gadX* mRNA fragment (mRNA) incubated at 37 °C for 1 h with the indicated combinations of *in vitro* synthesized GadY sRNA (GadY), crude cell extract from the GSO431 *rnc* mutant (protein), and heat-treated mutant crude cell extract (HT protein).

GadY (Fig. S2). Taken together, these results indicate that RNase III, in conjunction with any of the three forms of GadY, processes the *gadX* mRNA. The 140-nt 5′ fragment generated is common for all three forms of GadY, while the 3′ fragments were specific to each form of the GadY RNA.

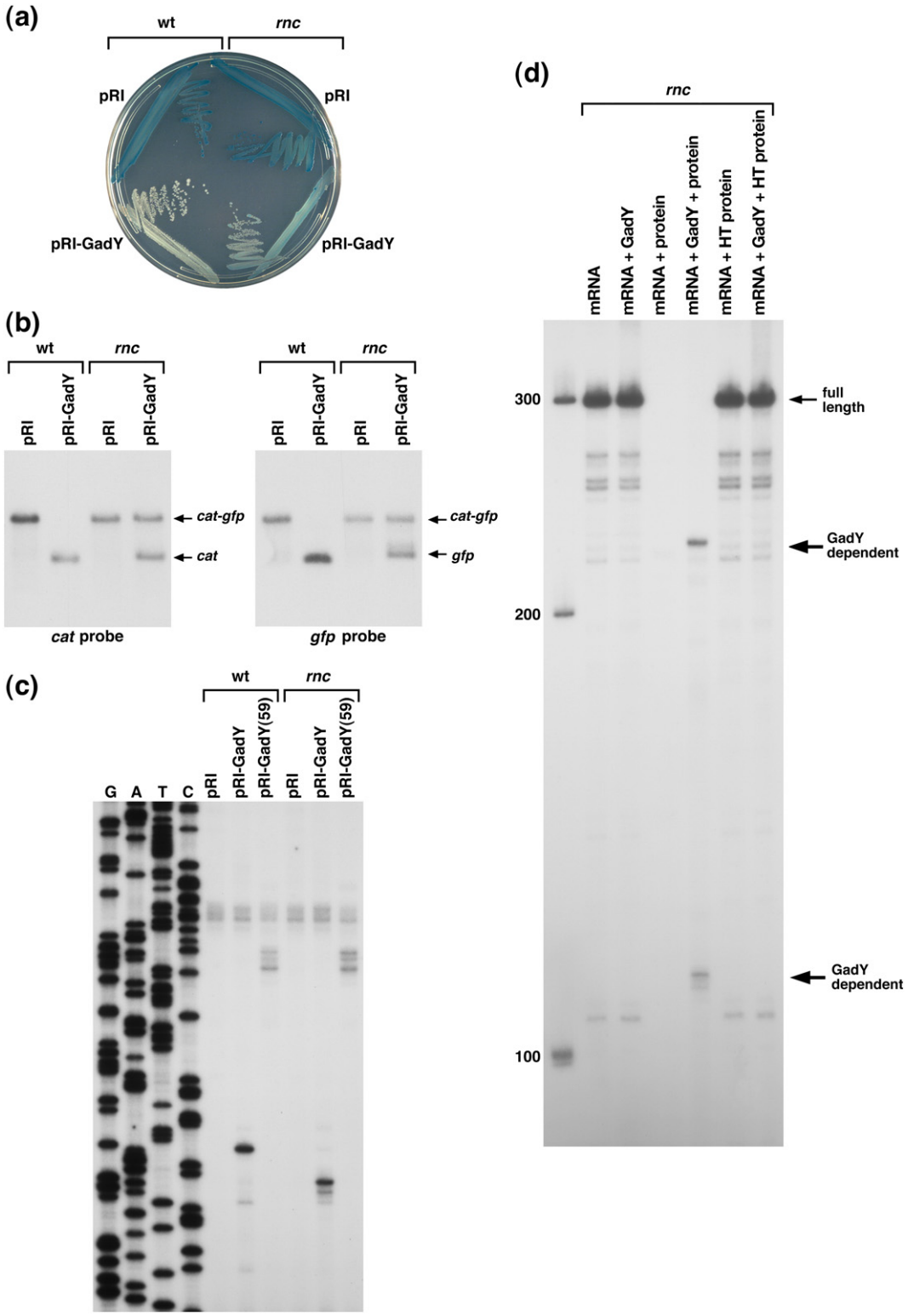

Fig. 7 (*legend on previous page*)

Processing detected in the *rnc* mutant strain

In Fig. 4, we noted that the *rnc* (RNase III) mutant strain had reduced levels of one of the full-length GadY-dependent bands. However, this mutant did not abolish processing, indicating redundancy with respect to enzymes capable of catalyzing GadY-directed processing. We further confirmed these results by testing for processing of the *lacZ* reporter in the *rnc* mutant strain on X-gal indicator plates (Fig. 7a). As also seen in Fig. 2b, colonies of the wild-type strain overexpressing GadY were white on the plates, demonstrating efficient processing of *lacZ*. In the absence of GadY, the colonies were dark blue, consistent with a lack of processing. Overexpression of GadY in the *rnc* mutant strain resulted in colonies that were pale blue, indicating that processing still occurs, although slightly less efficiently than in the RNase III-proficient strain.

We also examined the processing of the *cat-gfp* reporter construct by Northern blot analysis (Fig. 7b). Again, GadY-dependent processing was still observed. However, some differences were noted. While the full-length *cat-gfp* transcript was completely eliminated when GadY was overexpressed in the wild-type strain, approximately equal amounts of the *cat-gfp* transcript (~1.7 kb) were detected in the presence and absence of GadY overexpression in the *rnc* mutant. In addition, the *cat* transcript resulting from GadY-dependent processing migrated at a higher molecular weight (~0.85 kb) for RNA extracted from the *rnc* mutant strain. Using 3′ RACE, we determined that the *cat* transcript was ~20–50 nt longer in the *rnc* mutant strain compared to the wild-type strain (Fig. S3). Primer extension analysis also showed that the 5′ end of the *gfp* transcript was ~10 nt shorter in the *rnc* mutant when the full-length GadY was overexpressed, but remained the same when GadY(59) was overexpressed (Fig. 7c and Supplementary Fig. S3). Thus, although processing of the *cat-gfp* transcript is still GadY dependent and occurs in the region of base pairing in the *rnc* mutant strain, the 5′ and 3′ ends are different, indicating that the processing sites have shifted.

Finally, we prepared extracts from the *rnc* mutant strain and examined GadY-directed processing *in vitro*. Again, heat-sensitive, GadY-dependent processing was observed in the protein extract (Fig. 7d). As for the *in vivo* assays, the products observed for the *rnc* mutant extracts were of somewhat different size than the products observed for the wild-type extracts. A different subset of 5′ ends was also mapped by primer extension analysis (Fig. S4). Taken together, these results indicate that a nuclease, in addition to RNase III, is responsible for GadY-directed *gadX-gadW*-dependent cleavage, and that this enzyme cleaves at different nucleotides. We have not yet been able to identify this endonuclease despite attempts at genetic screens as well as biochemical purification.

Discussion

GadY is a member of a class of regulatory RNAs encoded on the DNA strand opposite their target RNAs. Initially, these *cis*-encoded RNAs seemed to be most abundant in extrachromosomal elements such as plasmids and bacteriophage; however, an increasing number of antisense sRNAs are being found to be encoded by bacterial chromosomes. These *cis*-encoded RNAs can regulate gene expression by numerous mechanisms including inhibition and attenuation of transcription and modulation of mRNA stability and translation (reviewed in Refs. 3,10). In many cases, the sRNA is encoded in a region that overlaps the 5′ end of the regulatory target gene. Three examples of this are *symE*-SymR of *E. coli*, *copT*-CopA from plasmid R1, and *tnp* (RNA-IN)-RNA-OUT from the IS*10* transposon. In these examples, base pairing between the antisense RNA and the 5′ end of the target mRNA results in translational repression of *symE* and *tnp* as well as RNase III-dependent destabilization of *copT* and *tnp*.[11–13] Unlike SymR, CopA, and RNA-OUT, the *E. coli* GadY RNA is encoded such that the gene overlaps the intergenic region of a two-gene operon and is one of only a few known cases where the sRNA is encoded opposite the middle part of a larger mRNA. Two other examples where a *cis*-encoded small RNA base-pairs internal to an mRNA are the OOP RNA from phage λ and RNAα from *Vibrio anguillarum* pJM1 plasmid.[14,15]

The genetic arrangement of GadY and *gadX-gadW* is most reminiscent of the OOP RNA and the *cII-O* operon of phage λ. The OOP RNA is transcribed from sequences opposite the intergenic region of the *cII-O* operon and extends to sequences opposite the 3′ end of *cII*. The OOP RNA was shown to be responsible for discoordinate regulation of the *cII* and *O* mRNAs.[14,16] This regulation is achieved by an OOP-dependent processing event mediated in part by the double-stranded endonuclease RNase III. This initial cleavage is followed by degradation of the upstream *cII* fragment, while the downstream *O* mRNA remains stable. We found that GadY acts similarly; base pairing of GadY with the *gadX-gadW* mRNA initiates processing mediated in part by RNase III. Processing directed by both OOP and GadY was shown to occur in regions of the target gene that are complementary to the sRNA.

The OOP and GadY RNAs also are similar in that they can direct the processing of the target mRNA by a mechanism that is RNase III independent. In both cases, the alternative processing sites are detected adjacent to the major RNase III processing sites in backgrounds lacking RNase III. This secondary

processing mechanism has not been elucidated for the OOP RNA. We speculate that the mechanism of RNase III-independent processing directed by OOP will be similar to the RNase III-independent processing directed by GadY. Our mutant analysis suggests that this additional ribonuclease may be an enzyme that has not yet been identified, since mutations affecting RNase E, RNase G, RNase BN, RNase P, the putative YbeY RNase,[17] as well as five of the endonucleases that are components of the type II toxin–antitoxin systems[18] did not affect GadY-dependent cleavage (Fig. 4, Fig. S1, and data not shown). Given that the levels of the RNase III-dependent band are increased in the *rne* mutant strain (Fig. S1), it is possible that the GadY-dependent cleavage involves a cascade of events. It is also conceivable that some of the observed GadY-dependent products arise when GadY base pairing protects *gadX* 3′ ends against the action of exonucleases. An important direction for future studies will be to identify the additional ribonuclease(s) involved in OOP- and GadY-dependent, RNase III-independent target mRNA cleavage.

GadY and OOP do differ in some respects. The OOP RNA partially overlaps the *cII* coding sequence at its 3′ end, whereas the overlap between GadY and *gadX-gadW* is contained completely within the intergenic region of the mRNA. This difference results in alternative outcomes for the target mRNA. Because OOP overlaps the *cII* coding sequence and processing was shown to occur in this region, the net outcome of OOP regulation is the downregulation of cII expression with the O expression levels remaining relatively stable. Negative regulation is also seen for the target of RNAα from *V. anguillarum*, where the sRNA overlaps a large portion of the coding sequence of *fatB*.[15] In contrast, GadY directs processing downstream from the *gadX* stop codon, most likely leading to increased stability of both the *gadX* half and *gadW* half of the mRNA.

We currently do not understand how the GadY-dependent processing event achieves this positive regulation, but some of our observations could be providing clues as to how the proposed stabilization might occur. Unprocessed *gadX-gadW* mRNA was nearly undetectable by Northern blot analysis and is likely to be unstable. The determinants for this instability may be contained within both *gadX* and *gadW*; the *cat-gadW* transcripts were more easily detectable by Northern blot analysis than the *gadX-gadW* mRNA, while the *cat-gfp* band was even more distinct (Fig. 1d and f and data not shown). GadY could remain base-paired at the processed 3′ end of *gadX* and block recognition of instability determinants present either directly at or some distance from the 3′ end, thus increasing the stability of the mRNA. Alternatively, GadY-directed cleavage could be leading to the removal of the instability determinants. Finally, the full-length *gadX-gadW* mRNA and the transcripts produced by GadY-dependent cleavage could fold into different secondary structures that have different susceptibility to degradation.

We predict that the GadY RNA will not be the only *cis*-encoded RNA that acts to increase the stability of a message. Most *cis*-encoded sRNAs that have been examined thus far either block the translation of their target and/or lead to the destabilization of the target. However, only a few chromosomal *cis*-encoded RNAs have been studied in detail, and numerous putative *cis*-encoded sRNAs are being discovered using deep sequencing techniques.[3] While further experimentation is needed, it is possible that some of these antisense RNAs will act similarly to the GadY or OOP RNAs in directing target mRNA cleavage.

Materials and Methods

Plasmids

All oligonucleotides and plasmids used in this study are given in Supplementary Tables S1 and S2, respectively. To generate pRI-GadY(90) and pRI-GadY(59), the corresponding regions of the *gadY* gene were amplified using PCR (using primers GadY-S1-gadY-A1 and JK117-JK118, respectively), digested with EcoRI and HindIII, and cloned into the corresponding restriction sites of pRI.[4] A second EcoRI restriction site at the +1 position of the P_{BAD} promoter of pBAD18-amp[19] was introduced by QuikChange mutagenesis (Stratagene) (using primers AZ784 and AZ785), generating pBAD-RI. The *gadY* regions corresponding to the full-length sRNA as well as the 90- and 59-nt fragments were amplified by PCR (using primers JK116-JK118, JK117-JK118, and GadY-S1-JK118, respectively), digested with EcoRI and HindIII, and cloned into the corresponding restriction sites of pBAD-RI to create pBAD-GadY, pBAD-GadY(90), and pBAD-GadY(59).

The *lacZ* gene was PCR amplified (using primers lacZ-S3 and lacZ-A3) and cloned into the BamHI and HindIII restriction sites of pACYC184[20] to create plasmid pACYC-*lacZ*. To introduce the region complementary to GadY, the entire pACYC-*lacZ* plasmid was amplified with primers lacZ-S10 and lacZ-A6. These primers hybridize directly adjacent to each other in the *lacZ* coding sequence and carry 6-nt in-frame extensions corresponding to the BglII and XhoI restriction sites, respectively. The 105-nt region corresponding to the *gadY* sequence was amplified, and a stop codon was eliminated by first amplifying two halves of the sequence overlapping at the position of the point mutation. The 5′ half of the sequence was PCR amplified with primers gadY-QC2 and gadX-S1, and the 3′ half of the sequence was PCR amplified with primers gadY-QC1 and gadW-A1. These two PCR fragments were fused by overlapping extension as described[21] using primers IG-S1 and IG-A2. The modified complementary GadY fragment was cloned into the BglII and XhoI

restriction sites of the pACYC-*lacZ* PCR fragment, creating a new plasmid named pACYC-$lacZ_{gadX}$.

Strains

All of the strains used in the study are listed in Supplementary Table S3. All of the reporter strains were generated using the mini-λ-red recombination method.[22] In all cases, the mini-λ-Tet^R located on the chromosome of NM500 (kind gift of N. Majdalani) was P1 transduced into the relevant strain.

For the *cat* substitution of *gadX*, the *cat* gene from pACYC184[20] was amplified by PCR (using primers Cat-S2 and PxCat-S1) and recombined into the *gadX* region of GSO109 carrying mini-λ-red. Chloramphenicol-resistant recombinants that no longer contained the mini-λ or that still retained the mini-λ were denoted GSO129 and GSO129-mini-λ-Tet^R, respectively. For the *gfp* substitution of *gadW*, the *gfpmut3.1* gene from plasmid pMG47[23] was amplified with primers gfp-S3 and gfp-A2. The *kan* gene from plasmid pKD4[24] was amplified with primers pKD4-S2 and pKD4-A2. The *gfp* DNA fragment and the *kan* DNA fragment were fused by splicing by overlapping extension as described[21] with primers gfp-S2 and PS2-X-A1 and inserted into strain GSO129-mini-λ-Tet^R, creating GSO403.

For the *lacZ* reporter strain, the modified *lacZ* gene from pACYC-$lacZ_{gadX}$ was PCR amplified with primers lacZ-S3 and lacZ-234-R. The *kan* resistance gene was amplified from pKD4 as above. The *lacZ* PCR fragment and *kan* PCR fragment were fused by splicing by overlapping extension as described[21] with primers GadX-LacUP and PS2-X-A1 and recombined into the *gadX* locus in strain NM700 (kind gift of N. Majdalani). The *kan* gene was removed by using the plasmid pCP20,[25] resulting in strain GSO404.

Strains carrying mutations in single genes encoding RNase activity were constructed by P1 transduction as follows. The *rnc*::Tn*10* allele from HT115[26] was transduced into GSO403 to give GSO432, and the *Δrnc*::*cat* allele from NB478[27] was transduced into GSO404 to give GSO405. To obtain the *rne* mutant strain, *zce-726*::Tn*10* linked to the *rne3071* allele from AC22[28] was transduced into GSO404. A transductant capable of growing at 43.5 °C, indicating that it retained the wild-type *rne* gene, was named GSO406. A transductant not able to grow at 43.5 °C, indicating that it received the linked *rne3071* temperature-sensitive mutation, was named GSO407. For the *rnpA* mutant strain, *zci-501*::Tn*10* linked to the *rnpA79* allele from strain NHY322[29] was transduced into GSO404. A transductant capable of growing at 43.5 °C, indicating that it retained the wild-type *rnpA* gene, was named GSO408. A transductant not able to grow at 43.5 °C, indicating that it received the linked *rnpA79* temperature-sensitive mutation, was named GSO409. For the *rng*, *rnlA*, and *elaC* mutants, the kanamycin resistance gene was amplified from pKD4[24] (using primers PS1-rng-PS2-rng, PS1-rnlA-PS2-rnlA, and mh686-mh687, respectively) and inserted into strain NM400 using the miniλ-red recombination method.[22] The corresponding *Δrng::kan*, *ΔrnlA::kan*, and *ΔelaC::kan* alleles were then P1 transduced into GSO404 to generate GSO410, GSO411, and GSO412, respectively.

The strains carrying mutations in multiple RNase genes were constructed as follows. The *Δrnc::cat* and *Δrng::kan* mutations were transduced into GSO406 and GSO407 to generate GSO413 (*Δrnc::cat zce-726*::Tn*10*), GSO414 (*Δrnc::cat zce-726*::Tn*10 rne*-3071), GSO415 (*Δrng::kan zce-726*::Tn*10*), and GSO416 (*Δrng::kan zce-726*::Tn*10 rne*-3071). The *Δrnc:cat* mutation was also transduced into GSO408 and GSO409 to generate GSO417 (*Δrnc:cat zci-501*::Tn*10*) and GSO418 (*Δrnc:cat zci-501*::Tn*10 rnpA79*), respectively. The *Δrng::kan* and *ΔrnlA::kan* alleles were transduced into GSO405 to generate GSO419 (*Δrnc:cat Δrng::kan*) and GSO420 (*Δrnc:cat ΔrnlA::kan*), respectively. The *rnc::Tn10* allele was transduced into GSO404 to generate GSO421. Subsequently, the *rnhA*::*cat* allele from MIC3009[30] was transduced into GSO421 to give GSO422 (*rnc*::Tn*10 rnhA*::*cat*).

Several triple-mutant strains were also constructed for this study. An *rnc rne rng* null strain was generated by tranducing the *Δrng::kan* allele into GSO413 and GSO414 to give GSO423 (*Δrnc::cat Δrng::kan zce-726*::Tn*10*) and GSO424 (*Δrnc::cat Δrng::kan zce-726*::Tn*10 rne*-3071). To generate the *rne rng elaC* strain, we first had to construct a *ΔelaC::cat* allele by amplifying the chloramphenicol resistance gene from pKD3[24] (using primers mh686-mh687) and inserting this into NM400 using the miniλ-red recombination method.[22] The *ΔelaC::cat* allele was then moved into GSO415 and GSO416 to generate GSO425 (*Δrng::kan ΔelaC::cat zce-726*::Tn*10*) and GSO426 (*Δrng::kan ΔelaC::cat zce-726*::Tn*10 rne*-3071). A two-step process was needed to generate an *rne elaC rnc* deletion strain. In the first step, the *ΔelaC::kan* allele was transduced into GSO406 and GSO407. Following this, the *Δrnc::cat* allele was moved into the two strains, giving rise to GSO427 (*zce-726*::Tn*10 ΔelaC::kan Δrnc::cat*) and GSO428 (*zce-726*::Tn*10 rne*-3071 *ΔelaC::kan Δrnc::cat*). Finally, to generate the *elaC rnc rng* deletion strain, the kanamycin cassette was removed from GSO412 (*ΔelaC::kan)* by using the plasmid pCP20.[25] The *Δrnc::cat* allele was then transduced into the resulting unmarked strain, followed by the introduction of the *Δrng::kan* allele, also via P1 transduction, giving rise to GSO429 (*ΔelaC Δrnc::cat Δrng::kan*).

The strains used for the biochemical studies were constructed as follows: GSO430 was generated by transducing the *ΔgadXW::kan* allele from strain EK442[5] into MG1655, and GSO431 was generated by transducing the *Δrnc::cat* allele from NB478[27] into GSO430.

RNA isolation

Total RNA was isolated as described.[31] Briefly, 700 μl of cell culture was added directly into 500 μl of 65 °C acid phenol containing 100 μl of 8% SDS, 320 mM sodium acetate, 16 mM EDTA (ethylenediaminetetraacetic acid). The cell lysate was extracted two times with 65 °C acid phenol (Ambion) and once with phenol–chloroform–IAA. RNA was ethanol precipitated and washed with 70% ethanol. RNA pellets were resuspended in DEPC–water.

RNA analysis

Primer extension and Northern blot analyses were performed as described.[4] Five micrograms of total RNA was used in all cases, except in the mapping of the processed ends of the native *gadX-gadW* mRNA, in which 30 μg of total RNA was used. Primers gadX-A1 and GadW-A2 were used to detect the wild-type *gadX-gadW*

mRNA; primers cat-A1 and Gfp-R were used to detect the reporter *cat-gfp* mRNA. Primers GadW-A2, lacZ-R2, and Gfp-R were used for mapping the 5′ ends in the context of the wild type, *lacZ* reporter, and *cat-gfp* reporter, respectively. 3′ RACE was carried out as described[32] using the following gene-specific primers: gadX-T7 to amplify the *gadX* mRNA, EF584 to amplify the *cat* mRNA, and EF607 to amplify the *gfp* mRNA. The cDNA products were cloned into pCRII TOPO (Invitrogen) and sequenced.

In vivo assay for GadY-dependent processing

Non-temperature-sensitive strains carrying pBAD-RI, pBAD-GadY, pBAD-GadY(90), and pBAD-GadY(59) were grown in LB medium containing 100 μg/ml amp and 1 mM IPTG at 37 °C until $OD_{600}=0.7$. GadY sRNA was induced by the addition of arabinose to a final concentration of 0.2%, and cells were incubated for an additional 20 min at 37 °C. For the temperature-sensitive mutant strains carrying the same plasmids, the cells were grown at 30 °C until $OD_{600}=0.7$. The cells were then shifted to 43.5 °C for 30 min to inactivate the mutant protein. GadY sRNA was induced by the addition of arabinose to a final concentration of 0.2% and incubated for an additional 20 min at 43.5 °C. In all cases, induction was stopped by the addition of 700 μl of cell culture to 500 μl of 65 °C acid phenol (Ambion), and total RNA was purified as described above. The 5′ ends of the processed RNAs were analyzed by primer extension analysis with ^{32}P-labeled primer lacZ-R2.

In vitro processing of *gadX*

The DNA template for the *in vitro* synthesis of full-length GadY was generated by PCR with primers GadY-T7 and GadY-T7-R. The DNA templates for the *in vitro* synthesis of GadY(90) and GadY(59) were generated with primers GadY90-T7 + GadY-T7-R and GadY59-T7 + GadY-T7-R, respectively. The DNA template for the *in vitro* synthesis of the *gadX* mRNA fragment was generated with primers GadX-T7 and GadX-T7-R. The three forms of GadY sRNA were synthesized by *in vitro* transcription using the PCR templates described above together with 100 U (2 μl) of T7 RNA polymerase (New England Biolabs) and unlabeled nucleotides (each at a 1.6 mM final concentration) in a 50-μl volume. The ^{32}P-labeled *gadX* mRNA fragment was similarly synthesized using the above PCR template; T7 RNA polymerase; unlabeled ATP, CTP, and GTP; and ^{32}P-UTP (Perkin-Elmer).

Protein lysate was generated by growing strain GSO430 or GSO431 to $OD_{600}=1.0$. Cells were harvested by centrifugation; resuspended in 50 mM Tris (pH 7.5), 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 200 mM KCl; and lysed by a high-pressure cell disruptor (Constant Systems Ltd.) at 27,000 psi. After lysis, the insoluble material was removed by centrifugation at 20,000*g*.

One microliter of ^{32}P-labeled *gadX* RNA and 5 μg of soluble protein lysate were incubated with or without 1 μl of GadY sRNA in 50 mM Tris (pH 7.5), 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 200 mM KCl (10 μl total volume) at 37 °C for 60 min. The reactions were stopped by the addition of phenol–chloroform–IAA and sodium acetate to a final concentration of 0.3 M. RNA samples were extracted and ethanol precipitated. RNA pellets were resuspended in formamide gel loading buffer. RNA samples were separated on a 6% acrylamide gel alongside a Perfect Marker RNA ladder (Novagen) and visualized by autoradiography. Processing of unlabeled *gadX* mRNA was performed identically, except the treated RNA was resuspended in DEPC-treated water after ethanol precipitation.

Purification of processing activity (RNase III)

All purification samples were tested for processing activity as described above. Cells from 2 L of GSO430 grown in 2 L of LB broth at 37 °C to $OD_{600}=1.0$ were harvested by centrifugation; resuspended in 20 ml of 50 mM Tris (pH 7.5), 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 20 mM KCl; and then lysed by a high-pressure cell disruptor (Constant Systems Ltd.) at 27,000 psi. The insoluble material was removed by centrifugation at 20,000*g* for 20 min. The protein lysate was subtractively purified on a HiTrap Q anion exchange column (GE Healthcare) by collecting the flow-through lysate. Ammonium sulfate was added to the protein lysate to a final concentration of 40% and incubated on ice for 60 min. The precipitated protein was removed by centrifugation at 20,000*g* and the supernatant was saved for further analysis. Ammonium sulfate was added to the remaining supernatant to a final concentration of 60% and incubated on ice for 60 min. The precipitated protein was harvested by centrifugation at 20,000*g*. The protein pellet was resuspended in 4 ml of 50 mM Tris (pH 7.5), 5% glycerol, 10 mM $MgCl_2$, 1 mM DTT, 20 mM KCl. The resuspended pellet was further purified on a 26/60 Hi Prep Sephacryl S-300 sizing column (GE Healthcare). Active fractions from the sizing column were pooled and purified on a HiTrap Heparin column (GE Healthcare) using a linear 20–300 mM KCl gradient. The active fractions from the Heparin column were pooled and further purified on a 26/60 Hi Prep Sephacryl S-300 sizing column (GE Healthcare). The active fractions from the sizing column were pooled and purified on a Mono S cation exchange column (GE Healthcare). At this final step, proteins were eluted with a linear 20–300 mM KCl gradient. Proteins from the active fractions were TCA precipitated and separated on a 10–20% Tris–glycine SDS-PAGE gel and visualized with GelCode Blue stain reagent (Thermo Scientific). Protein bands were excised from the gel and analyzed by mass spectrometry.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.12.009

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